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Multidrug-resistant *Salmonella typhimurium* and *Salmonella enteritidis* identified by multiplex PCR from animals

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Abstract

Antibiotic resistance in *Salmonella enteritidis* and *S. typhimurium*, one of the most frequent etiologic pathogens of food-borne bacterial gastroenteritidis in humans, is a serious health problem worldwide. Fifteen and 22 each of *S. enteritidis* and *S. typhimurium* were isolated from animals from 1983 to 1999 in Korea and tested for their antibiotic resistance patterns and phage types. *S. enteritidis* isolates were highly resistant to sulfonamides (86.7%) and four of them (26.7%) showed multiple antibiotic resistance. The most frequent phage type (PT) of *S. enteritids* was PT1 (33.3%) even though none of them had multiple antibiotic resistance. *S. typhimurium* isolates were highly resistant to streptomycin, sulfonamides, and tetracycline, 100%, 95.5%, and 86.4%, respectively. The incidence of multiple antibiotic resistance of *S. typhimurium* isolates was extremely high (100%) comparing to *S. enteritidis* isolates (26.7%). Two of the five ACSSuT type *S. typhimurium* isolates, resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, were phage type DT104. All *S. typhimurium* isolates were sensitive to florfenicol. For the rapid detection of multiple antibiotic resistant *S. enteritidis* and *S. typhimurium* isolates, particularly ACSSuT type *S. typhimurium* DT104, antibiotic resistance genes, *cmlA/tetR*, *PSE-1*, and *TEM*, and *Salmonella* spp. specific gene, *SipB/C*, were amplified using four pairs of primers in hot-started multiplex polymerase chain reaction. Two Korean isolates of *S. typhimurium* DT104 showed *TEM* amplicons instead of *PSE-1* for the ampicillin resistance. The multiplex PCR used in this study was useful in rapid detection of ACSSuT type *S. typhimurium* and identification of β-lactamase gene distribution among *Salmonella* isolates.

Key words: Multiple antibiotic resistance, *S. typhimurium*, *S. enteritidis*, multiplex PCR

Introduction

Salmonellae are wide spread in humans and animals worldwide and are of increasing public health concern as causative pathogens of food poisoning [12, 34]. While approximately 2,000 serotypes of *Salmonella* have been associated with enterocolitis, *Salmonella typhimurium* and *S. enteritidis* are two major etiologic agents of food-borne salmonellosis in humans [3, 6, 11, 14, 32]. *S. typhimurium* and *S. enteritidis* can colonize at the alimentary tract of animals without causing disease so that their contamination of human food chain can be significant health concern [16, 19]. Recently, increased level of antibiotic resistance of food-borne pathogens in human, such as *Salmonella*, *Campylobacter* and *Escherichia coli*, has been reported [2, 33, 36]. The multiple antibiotic resistance increased

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dramatically in *S. typhimurium* isolates, whereas the incidence of antibiotic resistance remained low in *S. enteritidis* isolates [8]. This phenomenon was mainly due to the spread of a multidrug-resistant epidemic strain of *S. typhimurium* definitive type (DT)104 with chromosomal integration of the genes encoding for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (ACSSuT type) [1, 4, 9, 18, 31]. Recent reports of increasing incidence of *S. typhimurium*, especially a multiple antibiotic resistant strain of *S. typhimurium* DT104, in humans and animals have posed a major emerging public health issue of international concern [5, 15]. Symptoms of illness caused by *S. typhimurium* DT104 are more severe and result in a higher mortality (3%) compared to other non-typhoid *Salmonella* infections (0.1%) [20] Most of the DT104 ACSSuT-type strains contain at least two integrons, one contains the aminoglycoside resistance gene cassette and the other contains a β-lactamase resistance gene cassette [4, 30]. A gene encoding sulfonamide resistance was found in the 3' conserved sequences of both integrons [30]. Integrons and gene cassettes related to resistance have been found in a wide range of bacterial pathogens, particularly Enterobacteriaceae, indicating inter-species horizontal gene transfer has occurred [27, 28, 29]. Thus, detection and monitoring of multidrug-resistant *S. typhimurium* and *S. enteritidis* is important to substantiate the choice of antibiotics for the treatment of clinical salmonellosis and to assess the risk of transfer of resistant genes to other bacterial pathogens [17]. In Korea, 23% of outbreaks of bacterial food poisoning from 1981 to 1990 were caused by *Salmonella* spp. [26]. And there have been continuing reports of Salmonellae-induced food poisoning caused mainly by *S. typhimurium* and *S. enteritidis* [13]. In this study, clinical isolates of *S. typhimurium* and *S. enteritidis*, 22 and 15 isolates, respectively, from various animal sources in Korea were tested to assess antibiotic resistance patterns and phage type prevalence. Antibiotic resistance genes were amplified using multiplex polymerase chain reaction (PCR) for rapid detection of multi-drug resistant *S. typhimurium*, particularly ACSSuT type *S. typhimurium* DT104, and *S. enteritidis*.

Materials and Methods

Bacterial strains

Eight *S. typhimurium* DT104 strains were obtained from Washington State University (Pullman, WA, USA). These strains were from fecal samples of cattle. Twenty-two *S. typhimurium* and fifteen *S. enteritidis* were isolated from animals during a last ten years (1989 to 1999) in Korea. Identification of the isolates was confirmed biochemically (Vitek system; bioMerieux, France), and strains were serotyped by slide agglutination and tube agglutination with *Salmonella* O and H group antisera, respectively (Difco Co., Detroit, MI, USA). All isolates were grown and maintained in nutrient broth (Difco) at 37℃.

Antibiotic resistance testing

The strians were tested for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, florfenicol, trimethoprim, enrofloxacin, norfloxacin, nalidixic acid, and ciprofloxacin, by the disk agar diffusion method performed on Muller-Hinton agar (Difco) plates. The antibiotic disks used in this study were purchased from Difco Laboratories unless otherwise specified. Disks contained the following amounts of antibiotic: ampicillin 10 μg (AM), chloramphenicol 30μg (C), streptomycin 10μg (S), triple sulfa 250μg (SSS; Becton Dickinson Cockeysville, MD, USA), tetracycline 30μg (TE), trimethoprim 5μg (TMP), florfenicol 30μg (FF; Handong, Seoul, Korea), enroflxacin 5μg (ENO), norfloxacin 10μg (NOR), nalidixic acid 30μg (NA), and ciprofloxacin 5μg (CIP). Inhibitory zones of the growth were measured and interpreted as per the criteria of the National committee for Clinical Laboratory Standards (NCCLS), except florfenicol was interpreted by the manufacture's instruction.

Phage typing

A total of 22 *S. typhimurium* and 15 *S. enteritidis* isolates were phage-typed with 30 and 15 bacteriophages, respectively, at National Institute of Health (NIH; Seoul, Korea).

Polymerase chain reaction

One or two colonies of *Salmonella* isolates grown on agar plates were resuspended in 500μℓ of distilled water and boiled (5 min) for DNA preparation. For more simplified procedure, some bacterial suspensions were used for multiplex PCR without boiling. Four pairs of primers, described by Carlson *et al.* [8], were used for the amplification of four individual target genes simultaneously. Sequences of oligonucleotide primers are listed in Table 1. Multiplex PCR was performed by using GeneAmp PCR system 2400 (The Perkin Elmer Corp., Norswalk, CT, USA) with a brief modification of S. A. Carlson *et al.* method. The reaction mixture consisted of 2.5μℓ of Mg-free 10×PCR buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl,

Table 1. Sequences of oligonucleotide primers

Primer	Sequence	PCR product(bp)	Accession number
SipB/C	5'-ACAGCAAAATGCGGATGCTT-3'(forward) 5'-GCGCGCTCAGTGTAGGACTC-3'(reverse)	232	U232561
cmlA/tetR	5'-CGCTCCTTCGATCCCGT-3'(forward) 5'-GCTGCGTTCATCTACAACAGAT-3'(reverse)	260	AF077555
PSE-1	5'-TTTGGTTCCGCGCTATCTG-3'(forward) 5'-TACTCCGAGCACCAAATCCG-3'(reverse)	132	M69058
TEM	5'-GCACGAGTGGGTTACATCGA-3'(forward) 5'-GGTCCTCCGATCGTTGTCAG-3'(reverse)	291	Y10281

0.1 mM EDTA, 1 mM DTT, 50% glycerol and 1% Triton X-100), 1$\mu\ell$ of 10 mM deoxynucleoside triphosphate mixture, 2.5$\mu\ell$ of 25 mM MgCl2, 2$\mu\ell$ of 50 pmol primer mixtrue (four pairs of primer), 3$\mu\ell$ of bacterial suspension, 1$\mu\ell$ (1 unit/$\mu\ell$) of Taq DNA polymerase (Promega; Madison, WI, USA), and 13$\mu\ell$ of sterile water. For more sensitive and specific amplification, hot-start method was used for multiplex PCR. Preincubation was at 95℃ for 5 min. Forty PCR cycles were run under following conditions: denaturation at 95℃ for 1 min, primer annealing at 48℃ for 30s, and DNA extension at 72℃ for 30s in each cycle. After the last cycle, the PCR tubes were incubated for 3 min at 72℃ and then 4℃.

Detection of amplified DNAs

Five microliters of the reaction products were electrophoresed in 3.0% agarose gels (Sigma; St. Louis, MO, USA) for 45 min at 100 V with 1.5$\mu\ell$ of 6× loading dye (Promega). PCR products were visualized by staining with ethidium bromide on a U.V. transilluminator.

Results

Antibiotic sensitivity testing

Of the 45 *Salmonella* isolates tested for resistance to antimicrobials, none of the isolates were resistant to enrofloxacin and norfloxacin. A total of 34 isolates (75.6%) were resistant to two or more antibiotics of which 32 isolates showed resistance to sulfonamides along with the resistance to one or more antibiotics. Korean isolates of *S. typhimurium* were highly resistant to streptomycin, sulfonamides, and tetracycline, 100%, 95.5%, and 86.4%, respectively. *S. enteritidis* isolates had high resistance only to sulfonamides (86.7%). All *S. typhimurium* isolates showed multiple antibiotic resistance patterns and were resistant to streptomycin and sulfonamides, except ST1 strain. The incidence of multi-drug resistance in *S. enteritidis* isolates was low (26.7%) compare to *S. typhimurium* isolates (100%). Eight isolates of *S. typhimurium* DT104 from WSU had same resistance patterns (Am Cm St Su Te Fc) and were resistant to florfenicol, whereas none of the *S. typhimurium* isolates from Korea showed resistance to florfenicol. Five Korean isolates of *S. typhimurium* (ST3, ST4, ST10, ST21, and ST22) showed penta-drug resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. In addition, ST21 strain showed additional resistance to trimethoprim. The results of the antibiotic resistance tests are given in Tables 2.

Phage types of *S. typhimurium* and *S. enteritidis* isolates

Five isolates of *S. enteritidis* (33.3%) were phage type 1 and were resistant to sulfonamides only. Two phage type 4 *S. enteritidis* isolates (13.3%) were susceptible to all antimicrobials tested, except sulfonamides. Phage types of 3, 21, and 9b were 13.3% (2 strain), 13.3% (2 strain), and 6% (1 strain), respectively. The most recent isolates of *S. typhimurium* were phage type DT104 and showed characteristic penta-resistance. ST5 and ST6 isolates were phage typed as 4 and 7, respectively. RDNC (Reaction Does Not Conformed) isolates had the lysotypes which were not consistent with the current phage-typing scheme. The results of phage typing are shown in Table 2.

Detection of resistance genes using multiplex PCR

Four pairs of primers were used for *SipB/C*, *cmlA/tetR*, *PSE-1*, and *TEM* amplification simultaneously in the multiplex PCR. Carlson *et al.* [8] used SipB/C, cmlA/tetR, and PSE-1 primer sets for the detection of *S. typhimurium* DT104 and substituted TEM primers for the PSE-1 primers in the modified multiplex PCR to detect other multi-resistant *Salmonella*, including ampicillin resistant *S.*

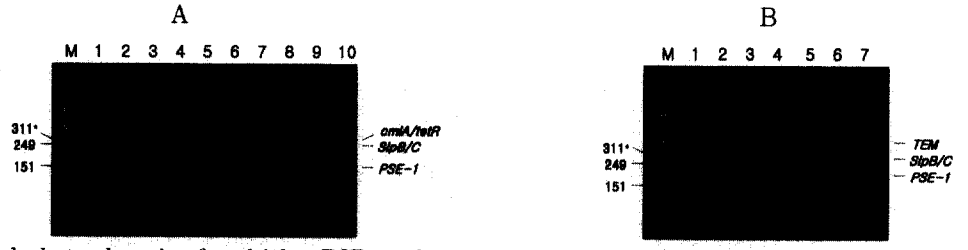

Fig. 1. Agarose gel electrophoresis of multiplex PCR products amplified from *S. enteritidis* isolates described in Table 2, using four pairs of primers. Lanes designated as M represent ϕX174 DNA/*Hinf* I markers (Promega). Lanes 1 used bacterial suspension without boiling in the multiplex PCR. A: Lane 1, DT2380; lanes 2-10, SE1-SE9. B: Lane 1, DT2486; lanes 2-7, SE10-SE15. *bp

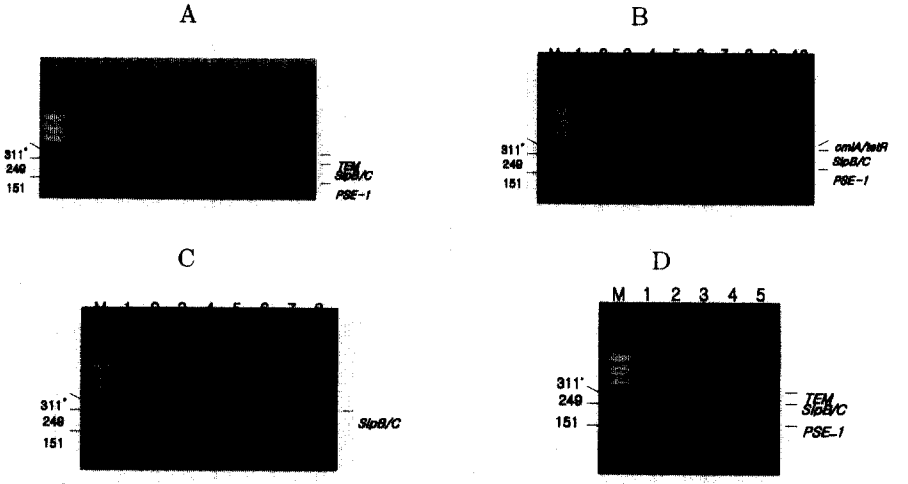

Fig. 2. Agarose gel electrophoresis of multiplex PCR products amplified from *S. typhimurium* isolates described in Table 2, using four pairs of primers. Lanes designated as M represent øX174 DNA/*Hinf* I markers (Promega). Lanes 1 used bacterial suspension without boiling in the multiplex PCR. A: Lane 1, DT2380; lane 2, DT2486; lane 3, DT2490; lane 4, DT2498; lane 5, DT2501; lane 6, DT2502; lane 7, DT2505; lane 8, DT2581; lanes 9-10, ST1-ST2. B: Lane 1, DT2490; lanes 2-10, ST3-ST11. C: Lane 1, DT 2498; lanes 2-8, ST12-ST18. D: Lane 1, DT2501; lanes 2-5, ST19-ST22. *bp

enteritidis and *S. typhimurium* U302 [8]. All multipex PCRs were hot-started for more specific and sensitive initial amplifications. For the more simplified procedure, some of the multiplex PCR were performed using bacterial suspension without boiling and produced target gene amplicons successfully (Fig. 1 and 2). Of 15 *S. enteritidis* isolates from Korea, SE12, SE13, SE14, and SE15 were resistant to ampicillin (26.7%) and only SE12 and SE15 had *TEM* gene conferring ampicillin resistance (Fig. 1B). Only *Salmonella*-specific *SipB/C* amplicon was observed in 13 other *S. enteritidis* isolates (Fig. 1A, B). Eight *S. typhimurium* DT104 isolates from WSU produced three amplicons for *SipB/C*, *cmlA/tetR*, and *PSE-1* genes, 250, 280, and 150 bp, respectively, whereas Korea isolates of *S. typhimurium* DT104 (ST21 and ST22) showed *SipB/C* and *TEM* amplicons (310 bp) (Fig. 2D). All other Korea *S. typhimurium* isolates produced single *SipB/C* amplicons, except ST3 and ST10 which had additional *TEM* genes (Fig. 2A, B, C, and D). No *cmlA/tetR* amplicon was detected from chloramphenicol resistant *S. typhimurium* isolated from Korea.

Discussion

Food poisoning and antibiotic resistance are two aspects of the threat *Salmonella typhimurium* and *S. enteritidis* pose to public health as major food-borne pathogens [15]. Antibiotic resistance, especially multiple antibiotic resistance, endows a selective advantage to pathogens exhibiting particular phenotypes [7, 35]. Increased multiple antibiotic resistance has been reported in *Salmonella* isolates, particularly in *S. typhimurium*, from various countries including Korea [10, 15, 25, 34]. *S. enteritidis* isolates used in this study had a relatively low incidence of multiple antibiotic resistance (26.7%) comparing to *S. typhimurium* isolates (100%). However, four *S. enteritidis*

Table 2. Antibiotic resistance patterns and phage types of *Salmonella* isolates

organism	Strain	Date & Source	Resistance pattern[a]	Phage type
S.enteritids	designation SE1	1995, layer	Su	RDNC[b]
S. enteritidis	SE2	1995, layer	Su	4
S. enteritidis	SE3	1995, broiler	St Su Te	9b
S. enteritidis	SE4	1996, layer	Su	4
S. enteritidis	SE5	1997, layer	Su	1
S. enteritidis	SE6	1997, layer	Su	1
S. enteritidis	SE7	1997, layer	Su	1
S. enteritidis	SE8	1997, layer	Su	1
S. enteritidis	SE9	1997, layer	Su	RDNC
S. enteritidis	SE10	1997, layer	Su	3
S. enteritidis	SE11	1998, layer	Su	1
S. enteritidis	SE12	1998, layer	Am Su	21
S. enteritidis	SE13	1999, layer	Am Na	3
S. enteritidis	SE14	1999, layer	Am St Su Te	RDNC
S. enteritidis	SE15	1999, layer	Am	21
S. typhimurium	DT2380	WSU[c], bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2486	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2490	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2498	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2501	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2502	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2505	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	DT2581	WSU, bovine	Am Cm St Su Te Fc	DT104
S. typhimurium	ST1	1983, unknown	St Te Na	RDNC
S. typhimurium	ST2	1985, unknown	*Cm*[d] St Su Te *Tr* Na	RDNC
S. typhimurium	ST3	1989, broiler	Am Cm St Su Te Na	RDNC
S. typhimurium	ST4	1992, swine	Am Cm St Su Te	RDNC
S. typhimurium	ST5	1992, swine	St Su Te	4
S. typhimurium	ST6	1992, swine	St Su	17
S. typhimurium	ST7	1992, quail	St Su Te	RDNC
S. typhimurium	ST8	1993, layer	St Su Te Tr	RDNC
S. typhimurium	ST9	1993, layer	St Su Te Tr	RDNC
S. typhimurium	ST10	1993, layer	Am Cm St Su Te	RDNC
S. typhimurium	ST11	1993, swine	St Su Te	RDNC
S. typhimurium	ST12	1994, swine	*St* Su Te	RDNC
S. typhimurium	ST13	1995, layer	St Su	RDNC
S. typhimurium	ST14	1995, swine	Cm St Su Te Tr *Na* Cp	RDNC
S. typhimurium	ST15	1995, swine	St Su Te	RDNC
S. typhimurium	ST16	1996, swine	St Su Te	RDNC
S. typhimurium	ST17	1996, swine	St Su Te	RDNC
S. typhimurium	ST18	1996, layer	St Su	RDNC
S. typhimurium	ST19	1997, duck	St Su Te	RDNC
S. typhimurium	ST20	1997, swine	St Su Te Cp	RDNC
S. typhimurium	ST21	1997, swine	Am Cm St Su Te Tr	DT104
S. typhimurium	ST22	1997, swine	Am Cm St Su Te	DT104

[a]Am: ampicillin; Cm: chloramphenicol; St: streptomycin; Su: sulfonamides; Te: tetracycline; Fc: florfenicol; Tr: trimethoprim; Na: nalidixic acid; Cp: ciprofloxacin.

[b]RDNC: reaction does not conformed to any recognized phage types.

[c]Washington State University.

[d]Antimicrobials shown in italics are for intermediate resistance.

isolates were multiple drug resistant and one of them was resistant to four antibiotics, which were ampicillin, streptomycin, sulfonamides, and tetracycline. The most common phage types (PT) of *S. enteritidis* are PT4 in Europe and PT8 and PT13a in the USA. These are associated with food poisoning and the concurrent widespread infection of commercial poultry meat and egg dishes [22, 23, 24]. Unlike in Europe and USA, although in a limited number of isolates, the most frequent phage type was PT1 (33.3%) in *S. enteritidis* isolated from Korea. There was no particualr correlation between phage types and multiple antibiotic resistance in these *S. enteritidis* isolates. Threlfall *et al.* reported a dramatic increase of multiple antibiotic resistance in *S. typhimurium* isolates which was related to the spread of a multi-resistant epidemic strain of *S. typhimurium* DT104, whereas the incidence of multidrug-resistant *S. enteritidis* remained low [33]. However, of the 22 multiple antibiotic resistant *S. typhimurium* isolated from Korea, only 2 isolates (ST21 and ST22) were phage type (DT) 104 indicating *S. typhimurium* DT104 is not the sole source of mutiple antibiotic resistance in *S. typhimurium* isolates. The most prevalent phage type of *S. typhimurium* isolates could not be assessed due to high incidence (81.8%) of RDNC strains. Only 4 isolates (ST5, ST6, ST21, and ST22) had lytic patterns corresponding to the current phage-typing scheme. Khan *et al.* described florfenicol resistance in *S. typhimurium* DT104 and used florfenicol resistance gene (*flost*) to detect *S. typhimurium* DT104 in multiplex PCR [21]. However, none of the *Salmonella* isolates from Korea exhibited florfenicol resistance, including two DT104 strains (ST21 and ST22), while all *S. typhimurium* DT104 from WSU showed resistance to florfenicol. Carlson *et al.* focused on the detection of ampicillin and chloramphenicol resistance genes to detect *S. typhimurium* DT104, since sulfonamide, streptomycin, and tetracycline resistance is overtly common in many *Salmonella* spp. [8]. And Casin *et al.* reported *PSE* (78%) and *TEM* (24%) genes as most prevalent β-lactamase genes among *S. typhimurium* isolates both from human and animal origins [9]. Based on these reports, we applied four primer pairs, SipB/C, cmlA/tetR, PSE-1, and TEM, for the amplification of the four genes simultaneously. All 15 *S. enteritidis* isolates produced *SipB/C* amplicons and SE12 and SE15 showed 310 bp *TEM* amplicons. Two other ampicillin resistant *S. enteritidis* isolates (SE13 and SE14) showed only the *Salmonella*-specific amplicon (*SipB/C*) which indicated the ampicillin resistance was conferred by other β-lactamase genes, such as *SHV*, and *OXA-2* genes [9]. Two Korea isolates of *S. typhimurium* DT104 (ST21 and ST22) were different from WSU *S. typhimurium* DT104 in that they produced *TEM* amplicon instead of *PSE-1* shown in all the WSU DT104 isolates in multiplex PCR. ST3 and ST10, ACSSuT type *S. typhimurium*, were also determined to have *TEM* genes for ampicillin resistance. One of ACSSuT type *S. typhimurium* (ST4), which was not DT104, showed no resistance-related amplicon. Of the 5 *S. typhimurium* isolates resistant to ampicillin, 4 isolates (80.0%) possessed *TEM* gene for the resistance phenotype.

In this study, incidence of multiple antibiotic resistance of *S. enteritidis* and *S. typhimurium* isolates was found in Korea, particularly all *S. typhimurium* isolates. The most recent isolates of *S. typhimurium* DT104 were determined to be ACSSuT-type *S. typhimurium* DT104 which could be a major cause of *Salmonella*-related enteritidis outbreaks in the near future in Korea. Since different pattern of possessing of resistance genes was shown between Korea *S. typhimurium* DT104 and US, further epidemiological studies are needed to analyze the difference in region. The multiplex PCR used in this study was useful in the rapid detection of ACSSuT type *S. typhimurium* and identification of β-lactamase gene distribution among *Salmonella* isolates. Monitoring of antibiotic resistance patterns of *S. typhimurium* and *S. enteritidis*, particularly emergence of *S. typhimurium* DT104, in farm animals remains critical importance due to the risk of resistance gene transmission to other pathogenic bacteria and transmission of these pathogens to human consuming products derived from these animals.

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